Euglena gracilis, A Test Organism for Study of Zinc[1, 2]

C. A. Price[3] & Bert L. Vallee

Biophysics Research Laboratory, Harvard Medical School &
Peter Bent Brigham Hospital, Boston, Massachusetts

Valuable information on the function of metals has been gained from studying metal deficiency states in a variety of organisms (8, 20). But our knowledge of the action of zinc in intact cells has not kept pace with the great strides made in the enzymology of zinc (20). This slow advance is due largely to methodological difficulties: organisms that have been used most frequently for enzymological studies are in general not suitable for trace metal studies. Conversely, studies on the nutrition and absorption of zinc and on the pathology of zinc deficiency have been conducted with organisms that have not usually lent themselves readily to biochemical studies (cf. 3).

In order to study zinc in vivo and in vitro with a single species, we have sought an organism in which A, the metal content could be rigorously controlled and B, which could be quantitatively disrupted, yielding a wide spectrum of highly active enzymes. *Euglena gracilis* apparently possesses these characteristics.

The present report documents the induction of a defined and controlled zinc deficiency in this alga.

Methods & Materials

Euglena gracilis (Klebs), "z" strain, was kindly provided by Dr. S. Hutner of the Haskins Laboratory, New York, N. Y.

► Special Chemicals. The iron, manganese, copper, and zinc sulfates employed in the growth media were Johnson and Mathey SpecPure salts. Ammonium glutamate was prepared from L-(+)-glutamic acid, Eastman Kodak. Metal-free water, ammonium hydroxide, and hydrochloric acid were prepared as previously described (18).

► Microchemical Analyses. Total nitrogen was determinated by acid digestion followed by Nesslerization (7). TCA-Soluble nitrogen was determined by treating cell samples with 4% trichloracetic acid (TCA) at 0 C for 1 hour, followed by centrifugation and analysis of nitrogen in the supernatant. TCA-Insoluble nitrogen was estimated by difference. TCA extractions of intact cells and of homogenates of intact cells yielded identical amounts of TCA-soluble nitrogen.

Zinc analyses of samples ashed in platinum dishes were by the dithizone method (21).

Copper was determined by the method of Peterson and Bollier (14), modified by the substitution of at least 0.5 ml of 2 M tris for saturated potassium phosphate; with this change the high concentrations of magnesium and iron in the cell samples remained in solution.

The spectrographic procedure and the flame spectrometry of potassium and sodium have been described (19, 22).

► Purification of Medium Components & Flasks. *Dithizone extraction.* Solutions of potassium phosphate, magnesium sulfate, calcium nitrate, and ammonium glutamate at the concentrations shown in table I were adjusted to pH 6 and shaken with successive small volumes of 0.01 % diphenylthiocarbazone (dithizone) in carbon tetrachloride until the ratio of $A_{625m\mu}$ to $A_{525m\mu}$ in the organic phase remained unchanged.

Ion exchange. Malic acid and sucrose solutions were freed from zinc by passage through a 30 × 300 mm column of AG 50WX8 resin, processed from Dowex-50 by the Bio-Rad Laboratories, Berkeley, Cal.

The Pyrex culture flasks were cleaned by soaking for at least 3 weeks in a 1:1 mixture of nitric acid and water. After each use they were returned to the nitric acid treatment until required.

► Growth Conditions. Media were dispensed in 1,000 or 1,500 ml volumes into 2-liter erlenmeyer flasks, autoclaved, and inoculated with exponentially growing cells. The flasks were placed on a rotary shaker, Model G10 of the New Brunswick Scientific Co., New Brunswick, N. J. in the dark at 21 to 25 C.

Cell concentration was measured by a modified turbidimetric procedure. Apparent optical density readings were first obtained with the Klett colorimeter on known dilutions of a cell suspension of arbitrary concentration. When the apparent optical densities were plotted against cell concentration, the readings were non-linear above Klett readings of about 40. In order to obtain a measurement that was a linear function of cell concentration, the linear part of the curve was extended. Values, called corrected Klett

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[3] Present address: Department of Plant Physiology, Rutgers—The State University, New Brunswick, N. J.

readings (K_c), could then be obtained from any given Klett measurement by reading off the extrapolated straight line immediately above the given measurement. For Klett measurements greater than 300 the correction became very large; in these cases the cell samples were diluted with water and the K_c obtained multiplied by the dilution factor.

The K_c values thus obtained are a linear measure of cell concentration (see fig 1).

Growth was monitored by removing samples sterilely and determining the K_c as described above.

► Harvest. The culture flasks were first cooled in ice. The contents were then transferred to 1-liter polyethylene centrifuge bottles. The cells were separated by centrifuging 1 minute at 1,000 rpm and at 0 C in the International Refrigerated Centrifuge, Model PR-2. The supernatant fluid was carefully siphoned off and the cells washed twice in metal-free distilled water.

For the various analyses, the cell concentrations of the final washed cell suspensions were usually adjusted to about 0.5 mg TCA-insoluble nitrogen per ml.

► Cell Breakage. Suspensions of exponentially-growing *Euglena* were passed three times through a Logeman Hand Mill (Fisher Scientific Co.) in the cold. Cell breakage was greater than 90 %. With cultures which had ceased growing, it was necessary to first freeze and thaw the cell suspensions in an acetone-dry ice bath, whereupon breakage in excess of 99 % was observed with all preparations.

Results

In examining *Euglena,* as a test organism for the induction of metal deficiencies the following characteristics were looked for: control of metal content, metabolic activity, ease of cell breakage, and homogeneity.

► Nutrition & Control of Metal Content. The medium of Hutner et al. (6) provided the basis for developing virtually zinc-free growth conditions. By deleting non-essential components, a still simpler medium was obtained (table I).

Those reagents found to contain zinc were purified either by extraction with dithizone or by ion exchange as described under Methods. The copper, ion, and manganese salts and the vitamins contained negligible amounts of zinc and were, therefore, used directly. The total zinc detected in the purified medium was equivalent to less than 0.2 μg per liter final volume.

The course of algal growth was monitored and the corrected Klett readings (K_c values) were found to be highly correlated with total cell nitrogen (regression line in fig 1). Independent of the amount of zinc present, a linear relationship between K_c and total cell nitrogen was found for all cells in the exponential phase of growth. For cells in the plateau phase of growth another linear function with different slope was found.

When 15 μmoles zinc per liter were included in the complete medium, the growth of the cultures, as measured by the K_c values, followed the kinetics of binary fission, $X = X_o e^{at}$, with $a = 2\ (\log_e 2) \cdot \text{day}^{-1}$. This reduced to a division time of 12 hours. The growth rate was independent of zinc concentration between 10^{-6} M and 3×10^{-5} M zinc in the medium (fig 2).

With sucrose or ethanol, extremely high cell densities were achieved, up to 400 mg cell nitrogen or about 3 g of dry weight per liter.

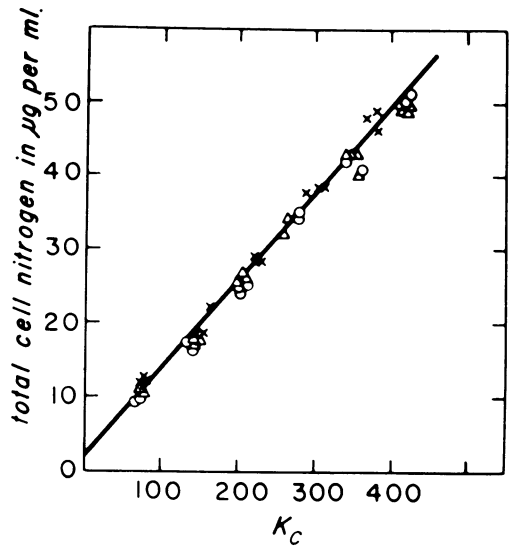
In the absence of added zinc, growth was greatly decreased, but high repeatability was achieved only when the culture flasks were specially purified as

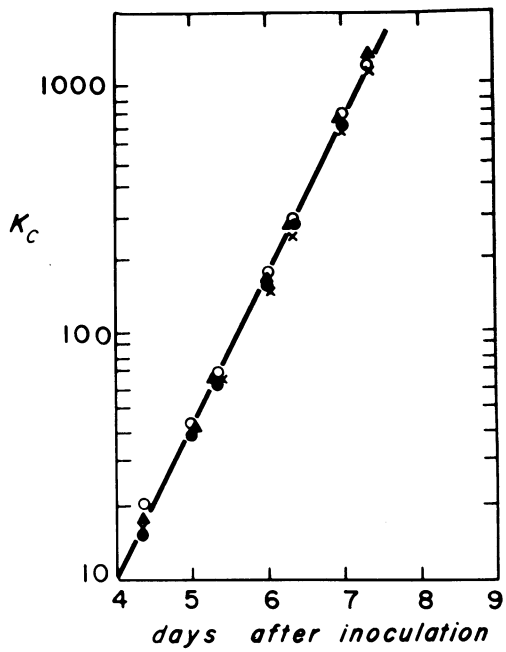
Table I

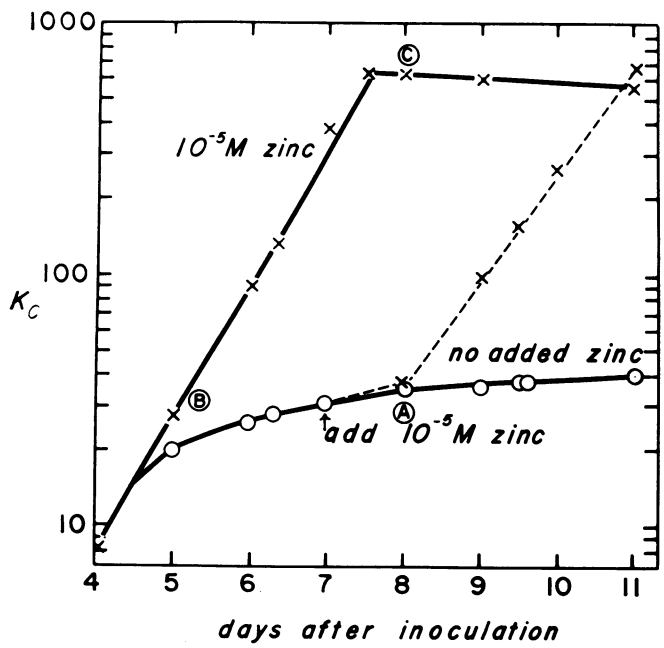
Composition of Complete Growth Medium*

Component	Stock solution		Final conc in medium	
	Conc (M)	Vol per liter medium (ml)	Wt per liter	Moles per liter
Potassium phosphate pH 6.0	1	2.0	87.6 mg K	2×10^{-3} in phosphate
Magnesium sulfate	1	1.62	40 mg Mg	1.6×10^{-3}
Calcium nitrate	0.95	0.1	4 mg Ca	9.5×10^{-5}
Iron sulfate	2×10^{-2}	1.8	2 mg Fe	3.6×10^{-5}
Manganous sulfate	10^{-2}	0.09	0.5 mg Mn	9×10^{-6}
Copper sulfate	10^{-2}	0.01	0.064 mg Cu	10^{-6}
Zinc sulfate	10^{-2}	1.5	1.0 mg Zn	1.5×10^{-5}
DL-Malic acid	1	2.0	270 mg	2×10^{-3}
Ammonium glutamate pH 6	1	0.4	3 g	2×10^{-2}
Sucrose	0.88	50	15 g	2.2×10^{-2}
or				
Ethanol	95(%)	3.25	3 g	6×10^{-2}
Thiamine. HCl	3.56×10^{-2}	5.0	60 mg	1.8×10^{-4}
Cyanocobalamin	10(μg/ml)	1.0	10 μg	7×10^{-9}

* Modified from Hutner et al. (6). Final pH adjusted to 3.5 with metal-free HCl.







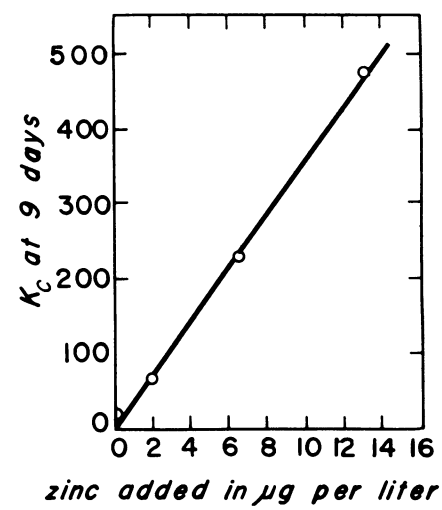


Fig. 1 (*Upper left*). Regression line of corrected Klett readings (K_c) of the washed cell suspension and total cell nitrogen. Zinc-sufficient cells harvested at 6 days (○) were in the exponential stage of growth; zinc-sufficient cells harvested at 7 days (×) were in early plateau stage, and zinc-deficient cells harvested at 6 days (△) were in early plateau stage.

Fig. 2 (*Upper right*). Independence of growth rate at higher zinc concentrations. Growth rate was followed as corrected Klett readings (K_c) at different zinc concentrations: ○, 10^{-6} M; ×, 3 $\times 10^{-6}$ M; ▲, 10 $\times$ 10^{-6} M, and ●, 30 $\times$ 10^{-6} M.

Fig. 3 (*Lower left*). Effect of adding limiting amounts of zinc on growth. Growth was followed as corrected Klett readings (K_c). The growth achieved in 9 days is plotted against the amount of zinc added.

Fig. 4 (*Lower right*). The recovery of growth rate upon the addition of zinc to deficient Euglena. Time course of growth as measured by corrected Klett readings (K_c) without added zinc (○), with 10^{-5} M zinc (×) added initially and after growth had essentially ceased. Points marked A, B, and C, show typical stages for harvest of zinc-deficient cells, exponential controls, and plateau controls, respectively.

Table II

Replication of Growth Obtained in Zinc Deficiency*

Grown in non-acid Soaked flasks	Grown in acid-soaked flasks		
Expt. 1	Expt. 2	Expt. 3	Expt. 4
83	30	23	22
44	30	21	25
38	31	22	24
44	32	21	21
89		21	22
375		20	21
		25	22
		22	21
			23
			21
Standard deviation	0.95	1.56	1.39

* Growth measured as corrected Klett readings (K_c) as described in text. Cultures were allowed to grow until zinc supply was exhausted. For comparison, when 15 μmoles zinc added per liter cultures reached 3,000 K_c.

described above (table II). Under these routine conditions the standard deviation of replicate cultures in the same experiment was less than two Klett units; this corresponded to a coefficient of variation of 6 %.

In figure 3 the final K_c achieved was plotted against the amount of added zinc. The relationship between growth and added zinc was linear except near the origin.

The response of a deficient culture to added zinc indicated that the cessation of growth with limiting zinc supply was due to a requirement for the metal rather than to the accumulation of toxic products.

Table III

Zinc Content of Cells Grown With & Without Added Zinc*

Expt.	Conc of added zinc (Moles/liter)	Growth phase	Zinc content of cells (μg per mg protein N)
1	1.5×10^{-5}	Exponential	18.0
			20.6
		Plateau	3.2
			3.2
	none	Plateau	0.80
			1.30
2	1.5×10^{-5}	Exponential	24.0
			23.2
	none	Plateau	0.68
			0.94
			0.80
			0.92
3	1.5×10^{-5}	Exponential	16.0
			17.3
	none	Plateau	0.74
			0.69

* All cells were grown with sucrose as principal carbon source. The duplicate zinc analyses were on single samples of cells.

The growth rate was rapidly re-established on addition of zinc as shown in figure 4.

The observed effects of zinc on Euglena growth, however striking, provided no information on the zinc level of the cells. In considering the metal content of zinc-deficient cells, two kinds of zinc-sufficient cells were taken for comparison: cells in the exponential and plateau stages. Zinc-deficient cells were harvested when growth had essentially ceased (point A, fig 4). Zinc-sufficient exponential stage cells were harvested at point B, when the *same amount of growth* had occurred and plateau stage cells were harvested at point C, the *same time* as the zinc-deficient cell harvest. In actual experiments (table III), analysis of the zinc-deficient cells showed about 5 % of the zinc of the exponential controls and between 8 and 20 % of the plateau controls on a TCA-insoluble nitrogen basis.

Table IV

Zinc Content of Cells Grown in Limiting Concentrations of Zinc*

Zinc added (in μg per liter)	Growth phase	Zinc content of cells (μg per mg protein N)
None	Plateau	0.49
		0.29
1.95	Plateau	0.31
		0.32
6.5	Plateau	0.34
		0.39
13	Plateau	0.31
		0.16
19.5	Plateau	0.49
		0.30
26	Plateau	0.40
		0.31
1,000	Exponential	6.8
		6.4
1,000	Plateau	3.9
		3.7

* All cells were grown with ethanol as principal carbon source. The duplicate zinc analyses were on single samples of cells.

The cells from the growth experiment presented in figure 3 were analyzed for zinc. The results, shown in table IV, show that all zinc-deficient cells have essentially identical zinc concentrations on a TCA-insoluble nitrogen basis.

Finally. it was necessary to establish that the cells were not suffering from some other metal deficiency in addition to or secondary to zinc; i.e., a conditioned deficiency. Ashed samples of two zinc-sufficient and one zinc-deficient cultures were therefore analyzed for their metal contents by emission spectrography, flame spectrometry, and microchemical analysis (table V). Zinc was the only metal that fell to a low concentration in zinc deficiency. The concentration of copper, however, increased tenfold over that in the exponential phase control.

Table V

Metal Content of Zinc-Sufficient & -Deficient Cells*

Culture	Metal content (μg metal per mg protein N)						
	Zn	K	Mg	Ca	Fe	Mn	Cu
Zinc-sufficient Exponential	6.6	79	33	1.4	13	0.50	0.96 0.92
Zinc-sufficient Plateau	3.8	60	30	1.9	7.2	0.82	0.35 0.35
Zinc-deficient Plateau	ND**	72	30	2.2	15	0.96	9.6 9.3

* Mg, Ca, Fe, Mn, and Zn were determined by spectrography; K by flame spectrometry, and Cu by microchemical analysis. Zinc-sufficient cells were grown in 1.5×10^{-5} M zinc; deficient cells were grown without added zinc. Ethanol was the principal carbon source.

** Microchemical analysis of similar samples gave ca. 0.3 μg Zn (table IV).

In order to determine if the medium provided a sufficient margin of safety with respect to metals, we compared the metal contents of the cells with the total metal content of the medium. With the exception of zinc, copper and iron, the concentration of metals found in the cells represented small fractions of the total metals added to the medium (table VI). Since iron and copper were non-limiting to growth at much lower concentrations (down to 3×10^{-7} M iron, zero added copper), it seemed most unlikely that any metal other than zinc was limiting growth in this system.

Table VI

Comparison of Metals Found in Cells With Amount Added to Medium*

Metal	Metal in cells from 1 l of medium (mg)	Metal initially added to medium (mg)	% Total metal in cells [Metal in cells / Metal added to medium × 100]
Potassium	9.1	87.6	10
Magnesium	4.5	40	11
Calcium	0.29	4	7.2
Iron	1.1	2	54
Zinc	0.57	1	57
Manganese	0.12	0.5	25
Copper	0.052	0.064	81

* Calculated for zinc-sufficient cells at plateau stage; ethanol as principal carbon source.

Nitrogen analyses brought to light another consequence of zinc deficiency: the percentage of the total nitrogen present at TCA-soluble nitrogen in zinc-deficient cells was twice that of zinc-sufficient exponential cells and nearly three times greater than zinc-sufficient plateau stage cells (table VII).

► Homogeneity of Cell Populations. Tables II, III, and IV and figure 2 indicate that different cultures were highly repeatable and reproducible with respect to growth rate, final yield, and cell zinc content.

Table VII

Effect of Zinc Level & Growth Phase on TCA-Soluble Nitrogen Fraction*

Zinc level of cells	Growth phase	TCA-Soluble Nitrogen as % total N		
		$\bar{x}$	s	n
Sufficient	Exponential	25.1	1.16	17
Sufficient	Plateau	17.9	1.60	7
Deficient	Plateau	51.2	10.4	15

* Sufficient cells were all grown in 1.5×10^{-5} M zinc; deficient cells include a range of limiting zinc concentrations from zero added to 3×10^{-7} M.

Discussion

Euglena gracilis has been examined for control of zinc content, ease of cell breakage, and homogeneity.

Judged by the criteria of growth rate, total growth achieved, and metal content, the cells can be made zinc deficient. The zinc concentration of Euglena can be controlled with a precision not previously reported in comparable studies of zinc deficiency in other organisms.

From the standpoint of growth or growth rates many organisms, including Euglena (12, 13), have been made zinc deficient (3), but with one notable exception (16), variability of results has limited their value as test organisms (e.g. 4, 5, 10, 11). In the present system the variation among replicate samples has been reduced virtually to the level of pipetting errors (cf. table II).

On a protein basis, zinc-sufficient Euglena contain up to 20 times higher concentrations of zinc than do zinc-deficient cells. While the zinc content of zinc-deficient cells is clearly independent of the amount of zinc initially present (table IV), it is also evident that the zinc content of sufficient cells may vary widely (tables III & IV). The use of metal content as a primary parameter of metal deficiency has not been universally recognized (1, 2).

Another necessary condition for true zinc deficiency (in contrast to a *conditioned deficiency* induced by zinc) is that other elements are not at limiting concentrations in the tissue. The data of table V indicate this condition is satisfied in Euglena with respect to potassium, magnesium, calcium, iron, manganese, and copper. No metal other than zinc is decreased to a low value. One metal, copper, is elevated in zinc deficiency, but the observed effects on growth are independent of added copper over a wide range. The cell contents of other metals and essential non-metals were not measured.

Only a few similar analyses of the metal composition of deficient organisms have been reported (9, 15, 17), and the results are difficult to interpret. In

order to compare the metal contents reported here with those determined on a dry weight basis, the approximate conversion factor of 40 mg dry weight per mg protein N may be used.

In conclusion these studies show that *Euglena gracilis* provides a unified system for precise, quantitative studies of zinc metabolism at the physiological, enzymological, and chemical levels of organization.

Summary

We have examined *Euglena gracilis* (Klebs) as a test organism for studying the metabolism of zinc. In the absence of added zinc the growth is reproducibly very low. The zinc contents of the harvested cells are as low as 5 % of the zinc-sufficient controls. Analyses of other nutrient metals in the cells indicate that zinc alone is limiting growth.

The cells, under relatively mild conditions, can be quantitatively disrupted.

We conclude that the alga is well suited for studying the biochemical consequences of zinc deficiency.

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